NATIONAL TOXICOLOGY PROGRAM
Technical Report Series
No. 442

TOXICOLOGY AND CARCINOGENESIS STUDIES OF *p*-NITROBENZOIC ACID

(CAS NO. 62-23-7)

IN F344/N RATS AND B6C3F$_1$ MICE

(FEED STUDIES)

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
National Institutes of Health

FOREWORD

The National Toxicology Program (NTP) is made up of four charter agencies of the U.S. Department of Health and Human Services (DHHS): the National Cancer Institute (NCI), National Institutes of Health; the National Institute of Environmental Health Sciences (NIEHS), National Institutes of Health; the National Center for Toxicological Research (NCTR), Food and Drug Administration; and the National Institute for Occupational Safety and Health (NIOSH), Centers for Disease Control. In July 1981, the Carcinogenesis Bioassay Testing Program, NCI, was transferred to the NIEHS. The NTP coordinates the relevant programs, staff, and resources from these Public Health Service agencies relating to basic and applied research and to biological assay development and validation.

The NTP develops, evaluates, and disseminates scientific information about potentially toxic and hazardous chemicals. This knowledge is used for protecting the health of the American people and for the primary prevention of disease.

The studies described in this Technical Report were performed under the direction of the NIEHS and were conducted in compliance with NTP laboratory health and safety requirements and must meet or exceed all applicable federal, state, and local health and safety regulations. Animal care and use were in accordance with the Public Health Service Policy on Humane Care and Use of Animals. The prechronic and chronic studies were conducted in compliance with Food and Drug Administration (FDA) Good Laboratory Practice Regulations, and all aspects of the chronic studies were subjected to retrospective quality assurance audits before being presented for public review.

These studies are designed and conducted to characterize and evaluate the toxicologic potential, including carcinogenic activity, of selected chemicals in laboratory animals (usually two species, rats and mice). Chemicals selected for NTP toxicology and carcinogenesis studies are chosen primarily on the bases of human exposure, level of production, and chemical structure. Selection *per se* is not an indicator of a chemical's carcinogenic potential.

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NTP TECHNICAL REPORT

ON THE

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P.O. Box 12233
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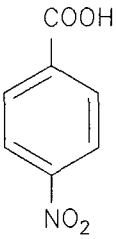
ABSTRACT



p-NITROBENZOIC ACID

CAS No. 62-23-7

Chemical Formula: $C_7H_5NO_4$ Molecular Weight: 167.12

Synonyms: 4-Nitrobenzoic acid; nitrodracylic acid; *p*-nitrobenzenecarboxylic acid; *p*-carboxynitrobenzene

p-Nitrobenzoic acid is produced in large volumes for organic synthesis and as an intermediate in the manufacture of pesticides, dyes, and industrial solvents. Groups of male and female F344/N rats and B6C3F$_1$ mice were exposed to *p*-nitrobenzoic acid (>99% pure) in feed for 14 days, 13 weeks, or 2 years for toxicity and carcinogenicity studies. Genetic toxicology studies were conducted in *in vitro* assays with *Salmonella typhimurium* and cultured Chinese hamster ovary cells, and in *in vivo* studies of erythrocyte micronucleus formation in mice in the 13-week study.

14-DAY STUDY IN RATS

Groups of five male and five female rats were given 0, 2,500, 5,000, 10,000, 20,000, or 40,000 ppm *p*-nitrobenzoic acid in feed for 14 days. All rats survived until the end of the study. Male and female rats given 20,000 and 40,000 ppm lost weight. The final mean body weights of 10,000, 20,000, and 40,000 ppm males were 82%, 60%, or 52% that of the controls, and the final mean body weights of 10,000, 20,000, and 40,000 ppm females were 87%, 68%, and 65% that of the controls. There were no clinical findings that were characteristic of organ-specific toxicity.

Absolute and relative spleen weights were significantly increased in rats exposed to 10,000, 20,000, and 40,000 ppm. There were decreases in erythrocyte count and hemoglobin and hematocrit values and increases in reticulocyte count, nucleated erythrocytes, and methemoglobin concentration that were most pronounced in the 20,000 and 40,000 ppm groups. Congestion of the spleen occurred in 10,000 ppm males and in 20,000 and 40,000 ppm females. Hypertrophy of the follicular epithelium of the thyroid gland was present in male and female rats exposed to 10,000, 20,000, or 40,000 ppm *p*-nitrobenzoic acid, while follicular hyperplasia was observed in the 40,000 ppm males and females. Atrophy of the testis was observed in 20,000 and 40,000 ppm males. Other lesions observed in 20,000 and 40,000 ppm rats included atrophy of the thymus in males and atrophy of the ovary, bone marrow, and thymus in females.

14-DAY STUDY IN MICE

Groups of five male and five female mice were given 0, 2,500, 5,000, 10,000, 20,000, or 40,000 ppm *p*-nitrobenzoic acid in feed for 14 days. Three males and two females given 40,000 ppm died during the study. All other animals survived until the end of the

study. Male mice given 20,000 and 40,000 ppm and females given 20,000 ppm lost weight. Mean body weight gains of 20,000 and 40,000 ppm males and 10,000, 20,000, and 40,000 ppm females were significantly lower than those of the controls. There were no clinical findings related to organ-specific toxicity although lethargy and ataxia were observed in 40,000 ppm mice.

Relative liver weights were significantly increased in 20,000 and 40,000 ppm males and females and in 10,000 ppm females. Absolute and relative thymus weights of 20,000 and 40,000 ppm males and of 10,000, 20,000, and 40,000 ppm females were reduced. No significant differences in hematology parameters occurred in exposed mice. Testicular degeneration was observed in three 20,000 ppm and two 40,000 ppm males. Bone marrow hemorrhage and atrophy occurred in 40,000 ppm females.

13-Week Study in Rats

Groups of 10 male and 10 female rats were given 0, 630, 1,250, 2,500, 5,000, or 10,000 ppm *p*-nitrobenzoic acid in feed for 13 weeks resulting in approximate daily doses of 40, 70, 160, 310, or 660 mg/kg to males and 40, 80, 170, 340, or 680 mg/kg to females. All rats survived until the end of the study. Mean body weight gains and final mean body weights were significantly less than those of the controls in 2,500, 5,000, and 10,000 ppm males and in 5,000 and 10,000 ppm females. There were no clinical findings related to organ-specific toxicity.

Differences in spleen weights and hematology parameters characteristic of regenerative anemia were observed in males and females, primarily in groups given 10,000 ppm. The absolute and relative spleen weights were significantly increased in 10,000 ppm males and females and the relative spleen weights were significantly increased in 5,000 ppm males and females. Methemoglobin, Heinz bodies, and reticulocyte counts were increased and erythrocyte counts, hemoglobin, and hematocrit values were decreased in 10,000 ppm males and females.

Congestion, pigmentation, and accumulation of macrophages in the spleen and pigmentation in the kidney occurred in 2,500, 5,000, and 10,000 ppm males. Congestion and pigmentation of the spleen occurred in 10,000 ppm females. A yellowish brown pigment (hemosiderin) in the spleen and kidney was associated with hemolytic anemia. Mild cytoplasmic hyaline droplet accumulation was present in renal tubule epithelial cells in 10,000 ppm males while karyomegaly was present in male and female rats exposed to 2,500, 5,000, and 10,000 ppm *p*-nitrobenzoic acid. A chemical-related testicular lesion, consisting of atrophy of the seminiferous tubules, occurred in 10,000 ppm males.

13-Week Study in Mice

Groups of 10 male and 10 female mice were given 0, 1,250, 5,000, 10,000, or 20,000 ppm *p*-nitrobenzoic acid in feed for 13 weeks resulting in approximate daily doses of 170, 330, 670, 1,900, or 4,000 mg/kg body weight to males and 240, 460, 970, 2,500, or 4,900 mg/kg to females. All mice survived until the end of the study, except one 1,250 ppm female that was killed accidentally. Final mean body weights and mean body weight gains of all exposed males and of 5,000, 10,000, and 20,000 ppm females were significantly lower than those of the controls. No clinical findings or differences in organ weights or histopathology related to organ-specific toxicity were observed in exposed mice.

2-Year Study in Rats

Groups of 60 male and 60 female rats were given 0, 1,250, 2,500, or 5,000 ppm *p*-nitrobenzoic acid in feed for 2 years. Ten males and 10 females from each exposure group were evaluated at 15 months.

Survival, Body Weights, Feed Consumption, and Clinical Findings

Two-year survival rates of 1,250 and 2,500 ppm males were similar to that of the controls. Two-year survival of 5,000 ppm males was marginally greater than that of the controls and was attributed in part to a decrease in the severity of nephropathy and a decrease in the incidence of mononuclear cell leukemia. Survival of exposed females was similar to that of the controls. Mean body weights of 5,000 ppm males were 2% to 8% lower than those of the controls through week 80. Final mean body weights of exposed males were similar to that of the controls. Mean body weights of 5,000 ppm females were 2% to 9% lower than those of the controls during the first year of the study and were 10% to 16% lower during the second year of the study. Final mean body weights of exposed females were 97% (1,250 ppm), 92% (2,500 ppm), and 84% (5,000 ppm) that of the

controls. Feed consumption by exposed males and females was similar to that by the controls. Dietary levels of 1,250, 2,500, or 5,000 ppm *p*-nitrobenzoic acid delivered approximately 50, 100, or 210 mg/kg body weight per day to males and 60, 125, or 250 mg/kg per day to females. There were no clinical findings attributable to organ-specific toxicity.

Pathology Findings

There were increases in the incidences of clitoral gland adenoma and of clitoral gland adenoma or carcinoma (combined) (4/50, 14/49, 15/49, 15/50) in exposed females. The incidences of clitoral gland adenoma or carcinoma (combined) in the exposed groups (29% to 31%) exceeded the historical control mean incidence (11%) and range (2% to 21%) in female F344/N rats in recent 2-year NTP feed studies. The increased incidences of clitoral gland neoplasms were considered to be some evidence of carcinogenic activity in female rats exposed to *p*-nitrobenzoic acid. The incidences of hyperplasia of the clitoral gland in exposed females were marginally lower than that of the controls (10/50, 6/49, 6/49, 7/50).

There was a chemical-related decrease in the severity of nephropathy in male rats. Male rat kidneys were examined using both single and step-section analyses, and the incidences of renal tubule neoplasms were not statistically greater than those of the controls. Mild hyaline droplet accumulation was observed in renal tubule epithelial cells in 10,000 ppm males in the 13-week study, but this effect was not severe enough to lead to a chemical-related neoplastic response in the 2-year study as has been observed with other chemicals.

At the 15-month interim evaluation, hematologic parameters characteristic of a mild regenerative anemia and significant differences in spleen weights were noted in 5,000 ppm females. These differences included decreases in erythrocyte count, hemoglobin, and hematocrit, increases in spleen weights, and hemosiderin accumulation in splenic macrophages.

At 2 years, significant decreases in the incidences of mononuclear cell leukemia were observed in 5,000 ppm males and 2,500 and 5,000 ppm females (males: 29/50, 35/50, 26/50, 2/50; females: 17/50, 11/50, 3/50, 0/50). While the mechanism for this decrease is unknown, decreases in the incidence of mononuclear cell leukemia have also been observed in 2-year studies with other amine/nitro compounds.

2-YEAR STUDY IN MICE

Groups of 60 male and 60 female mice were given 0, 1,250, 2,500, or 5,000 ppm *p*-nitrobenzoic acid in feed for 2 years. Ten males and 10 females from each exposure group were evaluated at 15 months.

Survival, Body Weights, Feed Consumption, and Clinical Findings

Two-year survival rates of exposed mice were similar to those of the controls. Mean body weights of 5,000 ppm males were 6% to 12% lower than those of the controls after week 17, and mean body weights of 5,000 ppm females were 12% to 24% lower than those of the controls after week 16. The final mean body weight of 5,000 ppm females was 19% less than that of the controls; final mean body weights of males were similar to that of the controls. Feed consumption by exposed mice was similar to that by the controls. Dietary levels of 1,250, 2,500, or 5,000 ppm *p*-nitrobenzoic acid delivered approximately 150, 300, or 675 mg/kg per day to males and 170, 365, or 905 mg/kg per day to females. There were no clinical findings of organ-specific toxicity. No chemical-related effects on hematology parameters were noted at the 15-month interim evaluation.

Pathology Findings

There were no increases or decreases in neoplasms in male or female mice that were considered to be related to chemical administration.

GENETIC TOXICOLOGY

p-Nitrobenzoic acid was mutagenic in *Salmonella typhimurium* strain TA100 with and without S9. No mutagenic activity was noted in strains TA98, TA1535, or TA1537, with or without S9. *p*-Nitrobenzoic acid induced sister chromatid exchanges and chromosomal aberrations in cultured Chinese hamster ovary cells in the absence of S9; with S9, results of both tests were negative. *In vivo*, no increase in micronuclei was observed in peripheral blood erythrocytes of male or female mice administered *p*-nitrobenzoic acid in dosed feed for 13 weeks.

CONCLUSIONS

Under the conditions of these 2-year feed studies, there was *no evidence of carcinogenic activity** of *p*-nitrobenzoic acid in male F344/N rats exposed to 1,250, 2,500, or 5,000 ppm. There was *some evidence of carcinogenic activity* of *p*-nitrobenzoic acid in female F344/N rats based on increases in the incidences of clitoral gland adenoma and of clitoral gland adenoma or carcinoma (combined). There was *no evidence of carcinogenic activity* of *p*-nitrobenzoic acid in male or female $B6C3F_1$ mice exposed to 1,250, 2,500, or 5,000 ppm.

There were chemical-related decreases in the incidences of mononuclear cell leukemia in exposed male and female rats. *p*-Nitrobenzoic acid caused mild hematologic toxicity in female rats.

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* Explanation of Levels of Evidence of Carcinogenic Activity is on page 10. A summary of the Technical Reports Review Subcommittee comments and the public discussion on this Technical Report appears on page 12.

Summary of the 2-Year Carcinogenesis and Genetic Toxicology Studies of *p*-Nitrobenzoic Acid

	Male F344/N Rats	Female F344/N Rats	Male $B6C3F_1$ Mice	Female $B6C3F_1$ Mice
Doses	0, 1,250, 2,500, or 5,000 ppm in feed (approximately 50, 100, or 210 mg/kg/day)	0, 1,250, 2,500, or 5,000 ppm in feed (approximately 60, 125, or 250 mg/kg/day)	0, 1,250, 2,500, or 5,000 ppm in feed (approximately 150, 300, or 675 mg/kg/day)	0, 1,250, 2,500, or 5,000 ppm in feed (approximately 170, 365, or 905 mg/kg/day)
Body weights	Dosed groups similar to control	High- and mid-dose groups lower than control	High-dose group lower than control	High-dose group lower than control
2-Year survival rates	12/50, 13/50, 13/50, 21/50	27/50, 23/50, 21/50, 21/50	39/50, 36/50, 39/50, 44/50	38/50, 36/49, 33/50, 30/50
Nonneoplastic effects	None	Mild hematologic toxicity	None	None
Neoplastic effects	None	Clitoral gland: adenoma (4/50, 12/49, 10/49, 12/50), carcinoma (1/50, 2/49, 5/49, 4/50), adenoma or carcinoma (combined) (4/50, 14/49, 15/49, 15/50)	None	None
Decreased incidences	Mononuclear cell leukemia (29/50, 35/50, 26/50, 2/50)	Mononuclear cell leukemia (17/50, 11/50, 3/50, 0/50)	None	None
Level of evidence of carcinogenic activity	No evidence	Some evidence	No evidence	No evidence
Genetic toxicology				
Salmonella typhimurium gene mutation:	Positive in strain TA100 with and without S9; negative in strains TA98, TA1535, and TA1537, with and without S9			
Sister chromatid exchanges				
Cultured Chinese hamster ovary cells *in vitro*:	Positive without S9; negative with S9			
Chromosomal aberrations				
Cultured Chinese hamster ovary cells *in vitro*:	Positive without S9; negative with S9			
Micronuclei in mouse peripheral blood cells:	Negative at 13 weeks			

EXPLANATION OF LEVELS OF EVIDENCE OF CARCINOGENIC ACTIVITY

The National Toxicology Program describes the results of individual experiments on a chemical agent and notes the strength of the evidence for conclusions regarding each study. Negative results, in which the study animals do not have a greater incidence of neoplasia than control animals, do not necessarily mean that a chemical is not a carcinogen, inasmuch as the experiments are conducted under a limited set of conditions. Positive results demonstrate that a chemical is carcinogenic for laboratory animals under the conditions of the study and indicate that exposure to the chemical has the potential for hazard to humans. Other organizations, such as the International Agency for Research on Cancer, assign a strength of evidence for conclusions based on an examination of all available evidence, including animal studies such as those conducted by the NTP, epidemiologic studies, and estimates of exposure. Thus, the actual determination of risk to humans from chemicals found to be carcinogenic in laboratory animals requires a wider analysis that extends beyond the purview of these studies.

Five categories of evidence of carcinogenic activity are used in the Technical Report series to summarize the strength of the evidence observed in each experiment: two categories for positive results (**clear evidence** and **some evidence**); one category for uncertain findings (**equivocal evidence**); one category for no observable effects (**no evidence**); and one category for experiments that cannot be evaluated because of major flaws (**inadequate study**). These categories of interpretative conclusions were first adopted in June 1983 and then revised in March 1986 for use in the Technical Report series to incorporate more specifically the concept of actual weight of evidence of carcinogenic activity. For each separate experiment (male rats, female rats, male mice, female mice), one of the following five categories is selected to describe the findings. These categories refer to the strength of the experimental evidence and not to potency or mechanism.

- **Clear evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a dose-related (i) increase of malignant neoplasms, (ii) increase of a combination of malignant and benign neoplasms, or (iii) marked increase of benign neoplasms if there is an indication from this or other studies of the ability of such tumors to progress to malignancy.
- **Some evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a chemical-related increased incidence of neoplasms (malignant, benign, or combined) in which the strength of the response is less than that required for clear evidence.
- **Equivocal evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing a marginal increase of neoplasms that may be chemical related.
- **No evidence** of carcinogenic activity is demonstrated by studies that are interpreted as showing no chemical-related increases in malignant or benign neoplasms.
- **Inadequate study** of carcinogenic activity is demonstrated by studies that, because of major qualitative or quantitative limitations, cannot be interpreted as valid for showing either the presence or absence of carcinogenic activity.

When a conclusion statement for a particular experiment is selected, consideration must be given to key factors that would extend the actual boundary of an individual category of evidence. Such consideration should allow for incorporation of scientific experience and current understanding of long-term carcinogenesis studies in laboratory animals, especially for those evaluations that may be on the borderline between two adjacent levels. These considerations should include:

- adequacy of the experimental design and conduct;
- occurrence of common versus uncommon neoplasia;
- progression (or lack thereof) from benign to malignant neoplasia as well as from preneoplastic to neoplastic lesions;
- some benign neoplasms have the capacity to regress but others (of the same morphologic type) progress. At present, it is impossible to identify the difference. Therefore, where progression is known to be a possibility, the most prudent course is to assume that benign neoplasms of those types have the potential to become malignant;
- combining benign and malignant tumor incidence known or thought to represent stages of progression in the same organ or tissue;
- latency in tumor induction;
- multiplicity in site-specific neoplasia;
- metastases;
- supporting information from proliferative lesions (hyperplasia) in the same site of neoplasia or in other experiments (same lesion in another sex or species);
- presence or absence of dose relationships;
- statistical significance of the observed tumor increase;
- concurrent control tumor incidence as well as the historical control rate and variability for a specific neoplasm;
- survival-adjusted analyses and false positive or false negative concerns;
- structure-activity correlations; and
- in some cases, genetic toxicology.

NATIONAL TOXICOLOGY PROGRAM BOARD OF SCIENTIFIC COUNSELORS
TECHNICAL REPORTS REVIEW SUBCOMMITTEE

The members of the Technical Reports Review Subcommittee who evaluated the draft NTP Technical Report on *p*-nitrobenzoic acid on June 22, 1993, are listed below. Subcommittee members serve as independent scientists, not as representatives of any institution, company, or governmental agency. In this capacity, subcommittee members have five major responsibilities in reviewing NTP studies:

- to ascertain that all relevant literature data have been adequately cited and interpreted,
- to determine if the design and conditions of the NTP studies were appropriate,
- to ensure that the Technical Report presents the experimental results and conclusions fully and clearly,
- to judge the significance of the experimental results by scientific criteria, and
- to assess the evaluation of the evidence of carcinogenic activity and other observed toxic responses.

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SUMMARY OF TECHNICAL REPORTS REVIEW SUBCOMMITTEE COMMENTS

On June 22, 1993, the draft Technical Report on the toxicology and carcinogenesis studies of *p*-nitrobenzoic acid received public review by the National Toxicology Program Board of Scientific Counselors Technical Reports Review Subcommittee. The review meeting was held at the National Institute of Environmental Health Sciences, Research Triangle Park, NC.

Dr. J.K. Dunnick, NIEHS, introduced the toxicology and carcinogenesis studies of *p*-nitrobenzoic acid by discussing the uses and rationale for study, describing the experimental design, reporting on survival and body weight effects, and commenting on chemical-related neoplastic lesions in female rats and nonneoplastic lesions in male (nephropathy) and female (hematologic toxicity) rats. Additional step-sections of the kidney were performed in male rats. The proposed conclusions were *no evidence of carcinogenic activity* in male F344/N rats, *some evidence of carcinogenic activity* in female F344/N rats, and *no evidence of carcinogenic activity* in male or female $B6C3F_1$ mice.

Dr. Brown, a principal reviewer, agreed with the proposed conclusions. He asked for comment on the seemingly paradoxical decrease in the incidence of mononuclear cell leukemia in exposed rats and the increased weight of the spleen. Dr. Ward noted that there was hematopoietic toxicity associated with the chemical and speculated that the stem cell in the bone marrow or spleen from which the leukemia derives may be one of the targets of the chemical resulting in an inhibition of leukemogenesis.

Dr. van Zwieten, the second principal reviewer, agreed with the proposed conclusions. He asked for substantiation of the conclusion that preputial gland and clitoral gland neoplasms were potentially lethal, because, in his experience, these neoplasms tend to be quite small and well circumscribed. Dr. S.L. Eustis, NIEHS, responded that the preputial gland neoplasms are not lethal in the sense of causing the animal's death, but as they get quite large with some becoming ulcerated, the animals are killed. Dr. J.K. Haseman, NIEHS, added that if a neoplasm were incidental, one would expect it to be more or less evenly distributed among the animals that died naturally and those that survived. However, in this study, the likelihood of observing a preputial gland neoplasm in an animal that died early was almost three times as high as in a surviving animal.

Dr. Ryan, the third principal reviewer, deferred her opinion of the proposed conclusions pending further discussion of exposure-related effects on clitoral gland and preputial gland lesions. She said there were inconsistencies in how body weight differences were discussed. For instance, decreased body weight in rats is offered as a possible explanation for the exposure-related decrease in leukemia. On the other hand, lack of an exposure-response for clitoral gland neoplasms was the main reason for some evidence rather than clear evidence for female rats, but was likely due, in her opinion, also to decreased body weight. Dr. Dunnick said the conclusion in female rats was based primarily on there being increases in neoplasms, mostly adenomas, at all three exposure levels. She agreed that body weight can affect the incidence of neoplasms, but the decrease in leukemia was believed to be more of a chemical effect than a body weight effect. Based on preputial gland neoplasms, Dr. Haseman said it was a close call between no evidence and equivocal evidence of carcinogenic activity in male rats. Dr. Eustis noted that the incidence of preputial gland carcinoma at the highest exposure level was within the historical control range.

Dr. Ward asked for comment on the presence of hyaline droplets in the kidneys of rats in subchronic studies and whether they were associated with $\alpha_{2\mu}$-globulin accumulation. Dr. Eustis said there was no evidence for accumulation of $\alpha_{2\mu}$-globulin in this study.

Dr. Brown moved that the Technical Report on *p*-nitrobenzoic acid be accepted with the revision discussed and with the conclusions as written for male rats and male and female mice, *no evidence of carcinogenic activity*, and for female rats, *some evidence of carcinogenic activity*. Dr. Taylor seconded the motion, which was accepted unanimously with ten votes.